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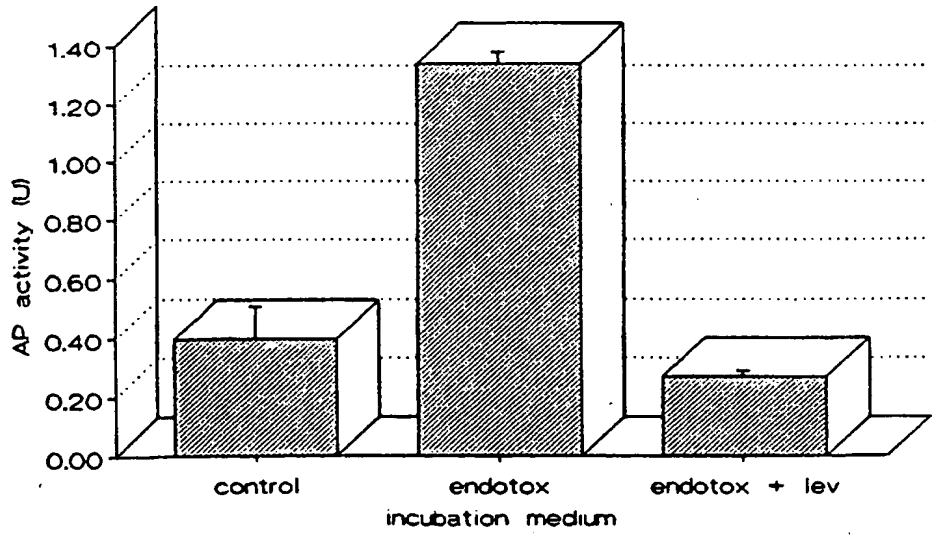


(57) Abstract

The invention relates to pharmaceutical compositions suitable for treating or curing clinical complications mediated by endotoxin, including sepsis. The compositions contain components suitable for detoxifying endotoxin rendering it less deleterious to mammals such as humans, in particular to patients with reduced host-defence resistance. The invention also relates to pharmaceutical compositions suitable for stimulating bone formation, e.g. form mending broken bone or for prophylaxis or therapy of osteoporosis and pharmaceutical compositions for decreasing or inhibiting undesired bone formation. The pharmaceutical compositions according to the invention are directed at modulating phosphatase activity *in vivo*.

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# PHARMACEUTICAL COMPOSITION COMPRISING PHOSPHATASE OR A DERIVATIVE THEREOF

## Field of the invention

The invention relates to pharmaceutical compositions suitable for treating or curing clinical complications induced by infections with Gram-negative bacteria, including sepsis. The compositions contain components suitable for detoxifying bacterial-wall derived lipopolysaccharides (also known as endotoxins) rendering these products less deleterious to mammals such as humans, in particular to patients with sepsis, optionally in combination with reduced host-defence resistance, i.e. after organ transplantations, during leucopenia (ref. 1) associated with cancer or chemotherapeutic treatment of cancer or during AIDS and AIDS-related diseases (ref. 2).

The invention also relates to pharmaceutical compositions suitable for stimulating bone formation, e.g. for mending broken bone or for prophylaxis or therapy of osteoporosis and also pharmaceutical compositions for decreasing or inhibiting undesired bone formation.

## Background information

Endotoxin is a negatively charged lipopolysaccharide present in the capsule of Gram-negative bacteria (ref. 3). Endotoxins are complexes of phospholipid (lipid A) and polysaccharide. The endotoxins produced by different bacteria differ in their antigenicity but they all have the same biological effects which are mainly due to lipid A. For the purposes of this description the term endotoxin also comprises enterotoxins. In addition to the negatively charged sugar moieties, an endotoxin contains two phosphate groups which are essential for its toxicity (ref. 3, 4).

Although it is an ubiquitous molecule in the external environment as well as in the gastro-intestinal tract of many species, an endotoxin can be extremely deleterious to these species once it leaves the gastro-intestinal tract e.g causing sepsis and inflammation such as in an abcess even in amounts as low as 10 picogrammes. Yet so far, no important endotoxin detoxifying mechanism has been found <u>in vivo</u> (ref. 5).

Endotoxin is known to induce serious even lethal complications (ref. 5 and 6). In fact, despite the use of antibiotics, this bacterial

product is the major cause of death in intensive-care units in Western society.

There are numerous different endotoxins produced by various microorganisms and consequently the actions of endotoxin in vivo are numerous as are the ways it can enter the organism. The symptoms associated with Gram negative infections therefore also vary widely among patients (ref. 7). These symptoms may be further complicated by septic shock of which hypotension, peripheral vasodilation and diffuse intravascular coagulation are the main characteristics (ref. 8). Subsequently organs such as heart (acute heart failure), lungs (adult respiratory distress syndrome), kidney (acute tubular necrosis) and brain may be affected (ref. 8). Endotoxin mediated pathology also comprises the syndrome of multiple organ failure and any other syndrome generally accepted in the art to be directly or indirectly caused by endotoxin.

To date, antibodies directed against endotoxin are the only endotoxin detoxifying proteins known to reduce toxicity irreversibly, but the clinical value of these antibodies remains to be established. Other substances which are able to bind endotoxin, such as lipopolysaccharide binding protein and high density lipoproteins (HDL) (ref. 9), exhibit the major drawback of forming reversible complexes in vivo. Upon dissociation of these complexes, the native (toxic) molecule is produced again. Furthermore although the detoxifying activity of plasma has been noted for some time (ref. 10) efforts to isolate or characterise the substance(s) responsible for this activity have not been successful. Other experimental approaches to treat sepsis include the application of preparations which antagonize the activities of cytokines (e.g. TNF-α), which are important mediators of endotoxin-induced shock, aggravating the effects of endotoxin in vivo. A major disadvantage of this approach is that these preparations do not detoxify the causative agent but rather block one of the reactions of the body to this toxin. In addition, antagonizing naturally occurring cytokines may cause multiple side- effects.

Alkaline phosphatase (EC 3.1.3.1) is a common enzyme present in many species, including man and has been studied extensively. The DNA sequence encoding alkaline phosphatase has even been obtained, but so far no commercial exploitation thereof has occurred. Although the enzyme is routinely applied as antibody label or as a marker for liver and neutrophil function, it's biological relevance is still unknown. Alkaline phosphatase is a membrane-bound ecto-enzyme which is known to dephosphorylate extracellular molecules. The enzyme is present in many organs, including

intestine, kidney, and neutrophils (ref. 11, 12 and 13). in vitro, it exhibits a pH optimum of approximately 10.5. (ref. 12). This extremely high pH optimum has hampered recognition of its biological relevance (ref. 12-14), because it was felt that this pH level does not occur in biological tissues of the intact organism.

Description of the invention

The subject of the present invention is based on our finding that alkaline phosphatase is endowed with phosphatase activity regulating certain vital body functions, even at physiological pH levels.

The basis for this insight was provided by the idea that at the molecular level in vivo, an alkaline micro-environment could present itself differently from aqueous solutions in vitro. In vivo negatively charged molecules may act as weak bases by their ability to bind $H^+$. Consequently these anions induce a local increase in the pH level thereby providing a micro environment with a pH value sufficient for the alkaline phosphatase to function as phosphatase.

The addition of negative charges can be provided by three different methods: addition of net negatively charged substrates to the enzyme, secondly providing the phosphatase with a membrane carrier with negative charges and thirdly by changing the ionisation of charged groups in the protein itself and/or introducing negatively charged moieties in the protein backbone or removing potentially positively charged groups in the protein.

These different mechanisms either alone or in combination may explain the unusual high pH optimum of the enzyme in vitro.

Similar considerations could be applicable to other types of phosphatases.

Another aspect of the present invention is based on the even more specific finding that alkaline phosphatase is also endowed with endotoxin detoxifying activity, even at physiological pH levels.

Endotoxin with it's negatively charged moieties can thus for example supply negatively charged residues necessary in the alkaline phosphatase's micro environment. In this way, this ubiquitous enzyme can provide protection in vivo against endotoxin, the ubiquitous product of Gram negative bacteria.

The use of enzyme preparations to detoxify endotoxin itself has the advantage that treatment of the disease in the early stage is possible and has the further advantage of irreversibly reducing toxicity of

the endotoxin. In addition as activity of enzymes is substrate specific the side-effects of using an enzyme such as alkaline phosphatase are limited.

Yet a further aspect of the invention is directed at the use of alkaline phosphatase for treatment or prophylaxis requiring increasing bone formation e.g. for stimulating the mending of broken bones or for treatment and/or prophylaxis of osteoporosis. The negative charges necessary for optimal alkaline phosphatase activity in vivo may not only be provided by the substrate but also by the micro-environment, or both.

In bone for instance, alkaline phosphatase is found in an extracellular environment rich in glycosaminoglycan (GAG)-chains, polysaccharides which are particularly characterized by their high content of negatively charged residues (ref. 15). To date, the role for alkaline phosphatase in bone formation is obscure. However, its significance may be reflected by the fact that the enzyme is present at sites of bone formation (e.g. in the extracellular space between two sites of a bone fracture (ref. 16 & personal observations). Moreover, a disease like hypophosphatasia, characterized by low levels of alkaline phosphatase activity in bone and serum is associated with skeletal deformations (ref. 17).

Again, the substrate for alkaline phosphatase is unknown, mainly because of the unphysiological high pH optimum of the enzyme using various substrates. However, in the light of our idea it is easily conceivable that when a given phosphorylated substrate becomes attached to the strongly negative GAG-chains, the proper micro-environment is created. In such a condition, alkaline phosphatase may express optimal activity at physiological pH levels, and dephosphorylate this substrate thereby contributing to the formation of insoluble calciumphosphate-complexes, an important constituent of bone matrices.

It may be speculated that lack of alkaline phosphatase activity may cause diseases like osteoporosis, frequently observed in elderly people. The higher incidence of osteoporosis in the female population can also be explained by this assumption since alkaline phosphatase activity is, at least in women, regulated by female hormones, as demonstrated by the rise in serum alkaline phosphatase activity during pregnancy (ref. 18). In the menopause this regulating mechanism disintegrates. Administration of alkaline phosphatase, or stimulating endogenous alkaline phosphatase activity and/or production, may therefore be beneficial to patients with osteoporosis or to persons with (multiple) bone fractures

and such treatment methods fall within the scope of the present invention. In addition, inhibition of alkaline phosphatase activity may be an option for therapy in patients with malignancies characterized by excessive bone-formation, such as osteosarcoma, or secundary tumours derived from metastatic carcinoma's. The high alkaline phosphatase activity found in osteosarcoma tissue (ref. 19) may therefore not only be a diagnostic marker for increased bone formation but an entrance for therapeutic intervention as well.

The subject invention is therefore also directed at a method of treatment of pathology associated with rapid bone formation such as osteosarcoma, said method comprising decreasing or inhibiting alkaline phosphatase activity, preferably in a target specific manner i.e. at the location where said pathology occurs. The decrease in phosphatase activity can be brought about e.g. by lowering formation of phosphatase or by competitively binding phosphatase preventing it's dephosphorylation. A pharmaceutical composition comprising at least one substance capable of decreasing or inhibiting phosphatase activity and/or the concentration of alkaline phosphatase (activity), said substance preferably being targeted to act at a location where undesired bone formation is to be prevented also falls within the scope of the invention.

To test the hypothesis that alkaline phosphatase is a protective enzyme of the host-defence system by its ability to detoxify lipopolysaccharides, we investigated whether alkaline phosphatase is able to dephosphorylate endotoxin of Escherichia Coli at physiological pH levels. Alkaline phosphatase activity was explored in 4% formalin-fixed cryostat sections (4 µm) of intestine, kidney and spleen according to standard histochemical methods at alkaline (ref. 20) and physiological pH levels (ref. 21), using either the conventional substrate ß-glycerophosphate (6.0 mg/ml) or endotoxin from Escherichia coli (0.55 mg/ml; serotype 0.55:B5, Sigma Chemical Co, St.Louis, U.S.A.). At alkaline pH level, the histochemical method of Gomori (ref. 20) was applied, whereas the method of Wachstein and Meisel (ref. 21) was used at the lower pH level. Sections were incubated with substrate for one hour at 37 °C. At pH 7.4 and 9.0, phosphate precipitates, indicating enzyme activity, were found in intestine and kidney sections when endotoxin was used as substrate (fig. 1). In the spleen, strong positive cells scattered throughout the red pulpa were found. Distribution of reaction product was identical for both substrates. All sections incubated without substrate were completely devoid of reaction product. In addition, the selective alkaline phospha-

tase inhibitor levamisole (1.0 mM) (ref. 20) completely inhibited phosphate release from endotoxin in kidney sections, whereas in intestine sections this activity was attenuated by the well known inhibitor of intestinal alkaline phosphatase, L-phenylalanine (5 mM) (ref. 22), but not by the stereoisomer D-phenylalanine (5 mM). Thus, both distribution of enzyme activity in various organs, as well as results obtained with selective inhibitors demonstrate that endotoxin is dephosphorylated by alkaline phosphatase at physiological pH levels.

The pH optimum of alkaline phosphatase activity was studied in a more quantitative way using tubular brushborder fragments of the rat kidney. This particular enzyme preparation has the advantage that it can be studied in association with the plasma membrane. Moreover, it can be completely inhibited by levamisole. Preparations of tubular brushborder fragments (isolated from the cortex of PVG rat kidneys using a sieve of 180 mesh and rinsed in 0.9% saline) which contained 12 µg protein (specific phosphatase activity: 86 U/mg, as assessed at pH 9.8) were added to 250 µl 2-amino-2-methyl-1,3-propanediol buffer of various pH levels. The buffer contained either endotoxin from E.Coli (1.25 mg/ml) or para-nitrophenolphosphate (0.5 mg/ml; pNPP). 2 mM $MgCl_2$ was added shortly before the start of the incubation period. After one hour incubation at 37 °C, inorganic phosphate concentrations were assessed according to the method of Chandrarajan (ref. 23). With the conventional substrate pNPP a steady increase in phosphate release along with the pH was observed (fig.2, upper left corner); however, when endotoxin was applied as substrate, enzyme activity reached a maximum at pH 8.8 and remained stable at this level. Endotoxin- as well as pNPP-dephosphorylation was inhibited by the alkaline phosphatase inhibitor levamisole (0.2 mM). Activity at high pH levels was not hampered by de-acetylation of fatty acyl chains of endotoxin occurring at alkaline conditions (ref. 24), since one hour pre-incubation of endotoxin at pH 9.8 did not inhibit dephosphorylation at pH 7.4 as tested histochemically. Thus, in contrast to the high pH optimum found with the substrate pNPP, alkaline phosphatase reaches maximal activity at a less extreme pH level when endotoxin is used as substrate. It may be speculated that the pH optimum is even lower when the enzyme is studied <u>in vivo</u> within its proper micro environment comprising the necessary additional negative charges to mimic the alkaline pH optimum observed <u>in vitro</u>.

To study the effect of alkaline phosphatase upon endotoxin toxicity, Limulus assays were performed. This Limulus assay is the stan-

dard method to assess endotoxin concentrations in vitro, based upon toxicity of the molecule towards the horseshoe crab *Limulus polyphemus* (ref. 25). Endotoxin (2.0 ng/ml) was incubated for one hour at 37 °C in RPMI-1640 buffer (pH 7.6), together with tubular fragments (0.8 µg protein/ml, specific phosphatase activity 86 U/mg). Control samples lacked either endotoxin or tubular brushborder fragments. Subsequently the Limulus assay was performed. Results show a significant reduction in endotoxin concentrations as measurable by this method in suspensions containing endotoxin and alkaline phosphatase activity, as compared to suspensions containing equal amounts of endotoxin without the enzyme (Table 1). It can be concluded that alkaline phosphatase is able to attenuate the toxicity of endotoxin molecules at physiological pH levels, as assessed in vitro.

**Table 1: Endotoxin concentrations as measured by the Limulus assay with and without pre-treatment with alkaline phosphatase**

| sample | [endotoxin] pg/ml |
|---|---|
| Endotoxin | 34.0 ± 13.0 |
| Endotoxin + alkaline phosphatase | < 0.05 ± 0 |
| Buffer | 11.3 ± 9.8 |

The toxicity of endotoxin treated with alkaline phosphatase was also studied in vivo, taking advantage of the fact that local inflammation following two successive endotoxin injections (the local Shwartzman-reaction (ref. 26)) can readily be quantified. If the detoxifying hypothesis were valid, this inflammatory reaction should be reduced after administration of endotoxin preparations pre-treated with alkaline phosphatase. Therefore, we elicited a local intradermal Shwartzman-reaction and treated the second endotoxin dose with tubular brushborder fragments at physiological pH. Influx of oxygen free radical producing cells, an important feature of the Shwartzman-reaction, was subsequently examined histochemically. Thus, the Shwartzman-reaction was elicited by two successive injections of endotoxin (from E. Coli 055:B5) divided by 20 hours

in female PVG rats (200 g). The first endotoxin injection (1 mg/kg b.w.) was administered intravenously, whereas the second injection consisted of an intradermal administration of a mixture of 70 µl RPMI 1640-medium (pH 7.6) supplemented with 2 mM $MgSO_4$ and 40 µg endotoxin (E) or $MgSO_4$ alone (C). Prior to injection, media were incubated (1 hour, 37°C) with 6 µg tubular brushborder fragments containing 86 U/mg alkaline phosphatase activity (A), with or without the alkaline phosphatase inhibitor levamisole (L; final concentration 1.0 mM). Control media were supplemented with saline (S) and lacked either endotoxin, or alkaline phosphatase, or both. Two hours after the intradermal injections, dermal sites were analyzed for influx of oxygen free radical producing cells, demonstrated histochemically with 3,3'-diaminobenzidine (DAB) at the light microscopical level (ref. 27). A significant influx of oxygen free radical producing cells was observed in dermal sites injected with untreated endotoxin as compared to controls (E/S versus C/S $p < 0.01$, Wilcoxon; fig 4). This inflammatory response was attenuated at dermal sites injected with endotoxin pretreated with tubular brushborder fragments (E/S versus E/A, $p < 0.05$, Wilcoxon), whereas endotoxin pretreated with tubular fragments plus levamisole displayed increased pro-inflammatory activity as compared to endotoxin pretreated with tubular fragments alone (E/A versus E/A/L, $p<0.025$, Wilcoxon). Each test was performed in duplicate on the same rat and results are expressed as the arithmetic means (+/- SD) of 6 rats. These data demonstrate that endotoxin treated with alkaline phosphatase exhibits reduced toxicity <u>in vivo</u> and that alkaline phosphatase may be able to detoxify endotoxin <u>in vivo</u>.

To examine the contribution of endogenous alkaline phosphatase activity in the endotoxin detoxification <u>in vivo</u>, we assessed the effect of levamisole upon endotoxin-sensitivity in rats, a species relatively resistant to Gram-negative lipopolysaccharrides. Female PVG rats, 6 months of age, received the alkaline phosphatase inhibitor levamisole (Sigma Chemical Co, St.Louis, USA) intraperitoneally (10 mg/kg b.w.), or saline at t=-24 and t=-1 hour. At t=0, rats received an i.v. challenge of 0.5 mg endotoxin, and blood was collected immediately prior to and at t=3, 6, 24 and 48 hours after this injection.

Serum glutamate-pyruvate transaminase activity, reflecting liver damage (an important pathogenetic factor in endotoxin-induced death) was assessed in these samples according to the method of Wroblewski and LaDue. Results showed no change in serum transaminase activity after treatment with levamisole alone as compared to saline treated rats

whereas an increase was found after the endotoxin challenge (fig.5). However, in contrast to the minor increase observed in rats receiving only endotoxin, rats pre-treated with levamisole displayed a very strong increment in serum transaminase activity ($p < 0.001$), demonstrating the involvement of endogenous alkaline phosphatase activity in the endotoxin-detoxifying activity of rats in vivo.

The anti-endotoxin activity being based on the dephosphorylating activity exhibited by alkaline phosphatase can naturally not be excluded for other phosphatases or derivatives of phosphatases. The subject invention is therefore directed at a pharmaceutical composition comprising at least a phosphatase or a vehicle capable of producing phosphatase as active component, said phosphatase having detoxifying activity for an endotoxin or a derivative of endotoxin having endotoxic activity and further comprising a pharmaceutically acceptable carrier. Use of a phosphatase as active component for the preparation of a pharmaceutical composition for prophylaxis or therapy of pathology mediated by endotoxin or a derivative of endotoxin having endotoxic activity is also covered by the subject invention. In particular a pharmaceutical composition comprising alkaline phosphatase or a vehicle capable of producing alkaline phosphatase forms a preferred embodiment of the invention. For use in a pharmaceutical composition the phosphatase must be obtainable in a substantially pure form. In general recombinant DNA techniques can provide a phosphatase suitable for use in a pharmaceutical composition according to the invention.

To date, four isozymes encoded by four distinct genes have been described. These include the intestinal form, the liver/bone/kidney-type (also present in neutrophils), the placenta-type, and the placental-like isozyme (present in germinal cells). Both the intestinal form and the liver/bone/kidney-type alkaline phosphatase exhibit endotoxin detoxifying activity, whereas there are no reasons to believe that the other isozymes of alkaline phosphatase are not able to degrade endotoxin. Therefore the subject invention comprises a pharmaceutical composition comprising any such isozyme.

The following case history illustrates the deleterious consequences of a reduced alkaline phosphatase activity in a mammal such as a human. A one year old female child suffered from recurrent endotoxaemia. These periods were accompanied with life-threatening symptoms of shock. Upon treatment, recovery was achieved, however, often followed by a relapse within a few weeks when endotoxaemia occurred again. In a period of

10 months, 12 relapses happened and finally the child died at the age of one year. The cause of death was diagnosed as septic shock induced by Gram-negative bacteria. The cause of the recurrent endotoxaemia itself was unknown. Our recent studies showed that in liver and spleen, alkaline phosphatase appeared normal, but enzyme activity was nearly absent in the ileum, an organ which normally expresses the highest enzyme activity of the human body. In the light of our finding, a reduced alkaline phosphatase activity in such a crucial organ, may explain the cause of death. It is easily conceivable that the lack of an endogenous detoxifying mechanism in the intestine results in recurrent endotoxaemia, considering the high content of E.Coli in the intestinal lumen. Such a deficiency might be adequately treated according to the subject invention.

A method for therapy or prophylaxis of pathology mediated by endotoxin or a derivative of endotoxin having endotoxic activity comprising administering to a subject a therapeutic amount of such a pharmaceutical composition therefore also falls within the scope of the invention.

Another aspect of the invention lies in the insight that in accordance with the Brønsted-Lowry classification of acids and bases, polyanions may act as weak bases since they are able to bind $H^+$. Thus extrapolating this to the fact that optimal functioning of proteins or polypeptides is often pH dependent and that in particular <u>in vitro</u> it has been illustrated that the incubation medium has to be alkaline for optimal activity of said proteins or polypeptides, in particular for alkaline phosphatase, we have concluded that <u>in vivo</u> polyanionic sites, for instance negatively charged sialoglycoproteins associated with cell membranes may meet the pH demands of such an enzyme or polypeptide.

Alkaline phosphatase is predominantly found in association with plasma-membranes. For example, neutrophils present the enzyme against the background of their negatively charged cell membrane instead of releasing it into the inflammatory micro-environment. For this reason it is felt that poly-anionic substrates could further contribute to favourable anionic conditions <u>in vivo</u> for phosphatase activity of phosphatases and derivatives thereof normally having an optimum at an alkaline pH, in particular for phosphatase activity of alkaline phosphatase.

If negatively charged sugar moieties of endotoxin influence the pH optimum of this enzyme activity, polycations could be expected to interfere with this reaction. Therefore, we treated the substrates with the cations poly-ethyleneimine (PEI) or poly-L-lysin (Lys). Substrates were preincubated for 30 minutes with either 0.5% PEI, 0.75% poly-L-lysin

or distilled water (C). Subsequently, incubations were carried out as described above and phosphate release was assessed. Both cations strongly affected dephosphorylation of endotoxin by neutralising the negative charges, whereas neither one of them significantly influenced pNPP degradation (fig. 3). The profound effect of PEI upon endotoxin degradation may be caused by neutralisation of negative charges whereas steric inhibition may also add to this effect. Interestingly, poly-L-lysin caused a shift of the pH optimum to a higher level, supporting the idea that negatively charged residues in the micro environment determine the pH optimum.

Additional support for the notion that negatively charged molecules in the micro-environment influence the pH optimum of alkaline phosphatase was also derived from experiments with intestinal enzymes. Histochemical assessment of the pH optimum of alkaline phosphatase in cryostat sections (4 µm) of rat intestine applying the method of Gomori with the substrate pNPP, revealed no significant change in staining intensity when the pH level of the incubation medium was varied from 7.8 to 9.8. However, alkaline phosphatase activity in serum, which is shown to be of intestinal origin, exhibits a pH optimum of 9.8 or higher. An important difference between intestinal alkaline phosphatase in situ and in serum is the sialoglycoprotein content thereof. Although the intestinal enzymes are embedded in sialated plasma membranes, serum alkaline phosphatase is not surrounded by these polyanions.

The subject invention is therefore also directed at a derivative of a phosphatase having phosphatase activity and comprising a higher content of negatively charged moieties than the corresponding part of native phosphatase. In particular the invention is directed at such a derivative being derived from alkaline phosphatase.

A derivative of a phosphatase according to the invention comprising a higher content of negatively charged moieties can comprise a higher content of derivatized alkaline amino moieties than the corresponding native phosphatase or the corresponding part thereof. This can for example be achieved by said derivative comprising a higher content of negatively charged N-acetylneuraminic acid groups (=sialic acid groups) than the corresponding native phosphatase or part thereof. Another possibility lies in the derivative comprising a higher content of negatively charged acid and/or reduced number of basic moieties than the corresponding native phosphatase or part thereof as such or in combination with the aforementioned embodiment. In yet another embodiment of the invention the

derivative of phosphatase can comprise a phosphatase moiety connected to a negatively charged protein or polypeptide. A suitable example of such a negatively charged protein is a modified negatively charged albumin, e.g. a succinylated albumin.

In a further aspect of the invention a derivative of phosphatase can comprise at least one modification for increasing the half life of said derivative in vivo, e.g. by preventing binding to galactose receptors, said modification e.g. being located at the terminal galactose residue of a phosphatase such as alkaline phosphatase. It was already known that removal of e.g.serum alkaline phosphatase is mediated by hepatic galactose receptors (ref. 28) but no attempt to modify a substrate of such a receptor has been taught or suggested previously. The modification can e.g. be the result of an oxidation or reduction.

The invention not only covers the derivatives as such in the various embodiments described above but also comprises combinations of the various aspects of such embodiments.

With regard to the various embodiments possible for a derivative of phosphatase WO 92/15316 gives examples of how to modify proteins and polypeptides in order to provide modified substances with an additional net negative charge by derivatisation of their amino groups and/or other basic functional groups with a reagent that prevents protonisation of basic amino groups and/or other basic functional groups or replaces said basic groups by one or more functional groups having a negative charge. The groups to be derived can be histidine and/or lysine residues. The reagent can be chosen from aldehydes, anhydrides, acid chlorides and isothiocyanates. For serum albumin a suitable reagent is cis-aconitate anhydride. Another suitable protein to be modified and linked to phosphatase to form a derivative according to the invention is α-acid glycoprotein.

It is also possible to create a derivative of alkaline phosphatase having optimal phosphatase activity at physiological pH, which derivative is therefore suitable for use in vivo and such a derivative also falls within the scope of the invention.

The subject invention not only covers the derivatives as described above in the various embodiments but also covers a pharmaceutical composition comprising at least one such derivative of phosphatase having phosphatase activity or a vehicle capable of producing such a derivative of phosphatase having phosphatase activity as active component and further comprising a pharmaceutically acceptable carrier.

In particular a pharmaceutical composition, wherein the active component as described i.e. the phosphatase, the derivative of phosphatase or a vehicle capable of producing phosphatase or a derivative thereof having phosphatase activity is embedded in the lipid bilayer of a liposome, preferably in combination with negatively charged membrane constituents is a preferred embodiment of the invention. In such a composition the phosphatase is a phosphatase having phosphatase activity having detoxifying activity for an endotoxin or a derivative thereof.

Use of a phosphatase derivative in any of the embodiments described above as active component for the preparation of a pharmaceutical composition for prophylaxis or therapy of pathology mediated by endotoxin or a derivative of endotoxin having endotoxic activity is also therefore covered by the subject invention. In particular a pharmaceutical composition comprising a derivative of alkaline phosphatase or a vehicle capable of delivering and/or inducing synthesis of alkaline phosphatase in any of the described embodiments forms a preferred embodiment of the invention.

A method for therapy or prophylaxis of pathology mediated by endotoxin or a derivative thereof having endotoxic activity comprising administering to a subject a therapeutic amount of such a pharmaceutical composition also falls within the scope of the invention. In particular the subject invention is also directed at a method for preventing occurrence of a pathology mediated by endotoxin or a derivative thereof having endotoxic activity following transplant or transfusion, said method comprising subjecting the material to be transplanted or transfused to treatment before and/or during and/or after transplant or transfusion with one of the following components:

- phosphatase having detoxifying activity for an endotoxin or a derivative of endotoxin having endotoxic activity, said phosphatase preferably being alkaline phosphatase, more preferably human alkaline phosphatase;
- a derivative of a phosphatase having phosphatase activity according to at least one of the embodiments of the invention as described above;
- a vehicle capable of delivering and/or inducing synthesis of a phosphatase having detoxifying activity for an endotoxin or a derivative of endotoxin having endotoxic activity, said phosphatase preferably being alkaline phosphatase;

- a vehicle capable of delivering and/or inducing synthesis of a derivative of phosphatase according to the invention as described above, as such or as active component of a composition.

To test whether this endotoxin detoxifying mechanism is upregulated in the presence of this bacterial product, human neutrophils were isolated, pre-incubated with endotoxin and assayed for alkaline phosphatase activity. Thus, neutrophils were isolated from normal human volunteers according to standard methods and collected in sterile medium. Neutrophils were not activated during the isolation procedure as assessed by measurements of superoxide anion production by these cells. Subsequently, cells ($0.9x10^7$ cells/ml) were incubated at 37 °C in buffer supplemented with endotoxin (20 pg/ml) or saline. After 30 minutes, alkaline phosphatase activity was assayed in these samples according to standard methods at pH 9.8 with pNPP as substrate. Phosphatase activity was measured with and without levamisole (1 mM) added to the medium. Results show an increase of 335% in neutrophilic alkaline phosphatase activity induced by endotoxin (fig. 6), which is in accordance with the proposed function of this enzyme.

The invention therefore also covers a pharmaceutical composition comprising at least one of the following components:
- a substance for stimulating phosphatase activity, in particular of a phosphatase or a derivative thereof having detoxifying activity for an endotoxin or a derivative of endotoxin having endotoxic activity, preferably for stimulating alkaline phosphatase activity;
- a vehicle capable of delivering and/or inducing synthesis of a substance for stimulating phosphatase activity, in particular of a phosphatase or a derivative thereof having detoxifying activity for an endotoxin or a derivative of an endotoxin having endotoxic activity, preferably for stimulating alkaline phosphatase activity as active component and further comprising a pharmaceutically acceptable carrier. The activating effect of the natural detoxifying action of alkaline phosphatase can thus be stimulated further and provide a means of defense against the negative pathological symptoms caused by endotoxins or derivatives thereof having endotoxic activity. In particular the substance for stimulating phosphatase activity, preferably for stimulating alkaline phosphatase activity can be selected from one or more of the following: an endotoxin, a substance having endotoxic activity, granulocyte colony stimulating factor (G-CSF), retinoic acid, a glucocorticoid and any other cytokines or substances known to stimulate phosphatase activity (ref. 13, 29 and 30).

The subject invention also covers the use of the aforementioned active components as active component for preparation of a pharmaceutical composition for prophylaxis or therapy of pathology mediated by endotoxin or a derivative of endotoxin having endotoxic activity.

A method for preventing occurrence of a pathology mediated by endotoxin or a derivative thereof having endotoxic activity, said method comprising activating endogenous alkaline phosphatase production in a subject by administering at least one of the aforementioned components as such or as active component of a composition to the subject also falls within the scope of the invention. As does a method for preventing occurrence of a pathology mediated by endotoxin or a derivative thereof having endotoxic activity following transplant or transfusion, said method comprising subjecting the material to be transplanted or transfused to treatment activating the endogenous production of alkaline phosphatase of said material by administering at least one of the following components:
- a substance for stimulating phosphatase activity of an endogenous phosphatase or a derivative thereof having detoxifying activity for an endotoxin or for a derivative of an endotoxin having endotoxic activity, preferably for stimulating alkaline phosphatase activity;
- a vehicle capable of delivering and/or inducing synthesis of a substance for stimulating phosphatase activity of an endogenous phosphatase or a derivative thereof having detoxifying activity for an endotoxin or for a derivative of an endotoxin having endotoxic activity, preferably for stimulating alkaline phosphatase activity as such or as active component of a composition to the subject. Another embodiment of the invention can be found in a method for increasing the presence of alkaline phosphatase, preferably endogenous alkaline phosphatase in the body, the tissue or the body fluid of an animal or human comprising application of at least one of the aforementioned components as such or as active component of a composition.

In summary from the above described experiments it can be concluded that enzyme preparations based upon alkaline phosphatase activity are able to dephosphorylate endotoxin thereby attenuating the toxicity of this molecule in vitro and in vivo. The physiological activity of this enzyme is most prominent when associated with negatively charged residues which provide the proper micro environmental conditions. Furthermore, alkaline phosphatase activity can be upregulated in cells of the host-defence system, providing a natural barrier against endotoxin.

The subject invention also covers a pharmaceutical composition comprising at least one of the following components:

- an alkaline phosphatase having phosphatase activity in or on bone in vivo;
- a derivative of said phosphatase, in particular a derivative having phosphatase activity according to any of the embodiments for derivatives according to the invention described above;
- a vehicle capable of delivering and/or inducing synthesis of said phosphatase and/or said derivative as active component and further comprising a pharmaceutically acceptable carrier. In vivo in this context implies in a macro environment having physiological pH, i.e. a pH of 7-8. A suitable embodiment of such a pharmaceutical composition comprises the active component embedded in the lipid bilayer of a liposome, preferably in combination with negatively charged membrane constituents.

The subject invention further comprises use of at least one of the following components:

- an alkaline phosphatase having phosphatase activity in or on bone in vivo;
- a derivative of said phosphatase in particular of a derivative of phosphatase having phosphatase activity according to any of the embodiments of a derivative according to the invention as described above in vivo;
- a vehicle capable of delivering and/or inducing synthesis of said alkaline phosphatase and/or said derivative as active component for preparation of a pharmaceutical composition for prohylaxis or therapy of pathology requiring increased bone formation such as stimulating mending of broken bones and prophylaxis or therapy of osteoporosis. Also a pharmaceutical composition comprising at least one of the following components:
- a substance for stimulating phosphatase activity and/or increasing synthesis of an alkaline phosphatase or a derivative thereof in vivo in or on the surface of bone;
- a vehicle capable of delivering and/or inducing synthesis of said substance for stimulating phosphatase activity as active component and further comprising a pharmaceutically acceptable carrier as such or in combination with the pharmaceutical composition described in the preceding paragraph falls within the scope of the invention.

The substance for stimulating alkaline phosphatase activity can be suitably selected from one or more of the following: an endotoxin, a

substance having endotoxic activity, granulocyte colony stimulating factor (G-CSF), retinoic acid, a glucocorticoid. Any other such substances known to a person skilled in the art can also be used to obtain the desired stimulation of alkaline phosphatase activity according to the invention.

Naturally a method for prophylaxis or therapy of pathology requiring increased bone formation such as stimulating mending of broken bone and prophylaxis or therapy of osteoporosis, said method comprising administering at least one of the following components:

- an alkaline phosphatase having phosphatase activity in or on bone in vivo;
- a derivative of an alkaline phosphatase having phosphatase activity according to claim 3 optionally in combination with any of claims 4-14 in vivo;
- a vehicle capable of delivering and/or inducing synthesis of said alkaline phosphatase or derivative thereof in vivo;
- a substance for stimulating phosphatase activity of an alkaline phosphatase and/or a derivative thereof having phosphatase activity in vivo in or on bone, said substance preferably being a substance for stimulating alkaline phosphatase activity;
- a vehicle capable of delivering and/or inducing synthesis of said substance for stimulating phosphatase activity in vivo as such or as active component of a composition to the subject falls within the scope of the invention.

A particular embodiment of this method comprises an active component wherein the substance for stimulating alkaline phosphatase activity is selected from one or more of the following: an endotoxin, a substance having endotoxic activity, granulocyte colony stimulating factor (G-CSF), retinoic acid, a glucocorticoid.

Figure legend

Figure 1

Alkaline phosphatase activity in cryostat sections of rat intestine (A) and kidney (B) as demonstrated at pH 9.0 with the substrate ß-glycerophosphate. Figures C and D show phosphatase activity in sections of intestine (C) and kidney (D) as demonstrated with endotoxin as substrate at pH 7.4. Significant dephosphorylation of endotoxin is found along intestinal crypts (fig. C) and in tubular brushborders of the kidney (fig. D), corresponding with the localization of alkaline phosphatase activity (fig. A & B). Furthermore, in intestinal sections this activity is reduced by addition of L-phenyl-alanine (fig. E), whereas endotoxin dephosphorylation in kidney sections is completely inhibited by levamisole (fig. F). Abbreviation: m = medulla. Magnification: 35x (A,B), 140x (C,E) and 56x (D,F).

Figure 2

Phosphatase activity, expressed as inorganic phosphate (Pi) release per hour, in suspensions of tubular brushborders at different pH levels with endotoxin as substrate. Phosphatase activity with the substrate para-nitrophenolphosphate (pNPP) is shown in the upper left corner. Tubular brushborder fragments were isolated from the cortex of PVG rat kidneys and added to 250 µl 2-amino-2-methyl-1,3-propanediol buffer at different pH levels, containing E. Coli endotoxin (1.25 mg/ml) or pNPP (0.5 mg/ml). 2 mM $MgCl_2$ was added immediately before incubation. Dashed lines indicate phosphatase activity in the presence of levamisole (0.2 mM). After one hour incubation at 37 °C, inorganic phosphate concentrations were assessed as described previously (ref. 29). Results are expressed as arithmetic means (± SD) of 6 assays, each assay was performed in duplicate. Results show that maximal dephosphorylation of endotoxin occurs at pH 8.8, whereas dephosphorylation of pNPP shows a steady increase to pH 9.8.

Figure 3

Phosphatase activity, expressed as phosphate (Pi) release per hour, in suspensions of tubular brushborders at different pH levels with endotoxin (fig. A) or pNPP (fig. B) as the substrate. Substrates were preincubated for 30 minutes with either 0.5% poly-ethyleneimine (*PEI*), 0.75% poly-L-Lysin (*Lys*) or distilled water (*C*). Subsequently, incuba-

tions were carried out as described at figure 2. Results are expressed as the arithmetic means of 4 assays, each assay was performed in duplicate (± SD).

Figure 4

To assess endotoxin toxicity in vivo, a localized Shwartzman-reaction (ref. 16) was elicited in the skin of PVG rats at different locations on the back. Prior to injection, all media were incubated (1 hour, 37°C) with 6 µg tubular brushborder fragments containing alkaline phosphatase activity (A), or with 0.9% saline (S). When indicated, the alkaline phosphatase inhibitor levamisole (L) was added (final concentration 1.0 mM). Two hours after the intradermal injections, dermal sites were analyzed for influx of oxygen free radical producing cells, demonstrated histochemically with 3,3'-diaminobenzidine (DAB) at the light-microscopical level[17]. Each test was performed in duplicate on the same rat and results are expressed as the arithmetic mean (± SD) of 6 rats.

Figure 5

Effect of endotoxin upon serum levels of glutamate-pyruvate transaminase (GPT) activity, which reflects damage of liver cells, in PVG rats. Part of the animals were pre-treated with levamisole (L; 10 mg/kg b.w.) at t=-24 and -1 hr. At t=0 rats received either 0.5 mg endotoxin from E.coli (E) intravenously or 0.5 ml saline. Results are expressed as arithmetic means (± SD of 4 rats) per group.

Figure 6

Effect of endotoxin upon alkaline phosphatase (AP) activity of human neutrophils. Neutrophils were isolated from whole blood according to standard procedures and incubated in 0.9 % saline with or without endotoxin from E.coli (20 pg/ml) for 30 minutes at 37°C. Subsequently, phosphatase activity of these cells was measured, using paranitrophenol phosphate as substrate at pH 9.8. Levamisole (1 mM) was added to confirm the involvement of alkaline phosphatase in phosphate release.

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CLAIMS

1. A derivative of a phosphatase said derivative having phosphatase activity and comprising a higher content of negatively charged moieties than the corresponding native phosphatase or the corresponding part thereof.

2. A derivative of a phosphatase having phosphatase activity, wherein said derivative has detoxifying activity for an endotoxin or a derivative thereof having endotoxic activity.

3. A derivative of a phosphatase having alkaline phosphatase activity in vivo in or on bone.

4. A derivative of phosphatase having phosphatase activity comprising at least one modification capable of increasing the in vivo elimination half life of said derivative e.g. by preventing binding of said derivative to galactose receptors, said modification e.g. being located at the terminal galactose residue of said derivative, said modification e.g. being the result of an oxidation or reduction.

5. A derivative of alkaline phosphatase exhibiting optimal phosphatase activity at physiological pH.

6. A derivative of a phosphatase according to any of the preceding claims, wherein said derivative has detoxifying activity for an endotoxin or a derivative thereof having endotoxic activity.

7. A derivative of a phosphatase according to any of the preceding claims comprising at least one modification capable of increasing the in vivo elimination half life of said derivative e.g. by preventing binding of said derivative to galactose receptors, said modification e.g. being located at the terminal galactose residue of said derivative, said modification e.g. being the result of an oxidation or reduction.

8. A derivative of alkaline phosphatase according to any of the preceding claims exhibiting optimal phosphatase activity at physiological pH.

9. A derivative of a phosphatase according to any of the preceding claims, said derivative being derived from alkaline phosphatase, preferably human alkaline phosphatase and preferably obtainable via recombinant DNA technology.

10. A derivative of a phosphatase according to any of the preceding claims, said derivative comprising a higher content of derivatized alkaline amino moieties than the corresponding native phosphatase or the corresponding part thereof.

11. A derivative of phosphatase according to any of the preceding claims, said derivative comprising a higher content of negatively charged N-acetylneuraminic acid groups than the corresponding native phosphatase or part thereof.

12. A derivative of phosphatase according to any of the preceding claims, said derivative comprising a higher content of negatively charged acid moieties and/or reduced number of basic groups than the corresponding native phosphatase or the corresponding part thereof.

13. A derivative of phosphatase according to any of the preceding claims, wherein the phosphatase moiety is connected to a negatively charged protein or polypeptide.

14. A derivative of phosphatase according to claim 13, wherein the phosphatase moiety is connected to a modified negatively charged albumin, e.g. to a succinylated albumin.

15. A pharmaceutical composition comprising at least one of the following components:
- a phosphatase having detoxifying activity for an endotoxin and/or for a derivative thereof having endotoxic activity, said phosphatase preferably being alkaline phosphatase, more preferably human alkaline phosphatase and said phosphatase preferably being recombinant phosphatase;
- a derivative of a phosphatase having phosphatase activity according to any of claims 1-14;
- a vehicle capable of delivering and/or inducing synthesis of said phosphatase and/or said derivative,

as active component and further comprising a pharmaceutically acceptable carrier.

16. A pharmaceutical composition according to claim 15, wherein the active component is embedded in the lipid bilayer of a liposome, preferably in combination with negatively charged membrane constituents.

17. Use of phosphatase and/or of a derivative of a phosphatase according to any of claims 1-14, said phosphatase preferably being alkaline phosphatase, more preferably human (alkaline) phosphatase and preferably being recombinant phosphatase, as active component for the preparation of a pharmaceutical composition for prophylaxis or therapy of pathology mediated by an endotoxin and/or by a derivative of endotoxin having endotoxic activity.

18. A method for therapy or prophylaxis of pathology mediated by an endotoxin and/or a derivative of endotoxin having endotoxic activity, said method comprising administering to a subject a therapeutic amount of a pharmaceutical composition according to any of claims 15 or 16.

19. A method for preventing occurrence of a pathology mediated by an endotoxin and/or by a derivative thereof having endotoxic activity, following transplant, implant or transfusion, said method comprising subjecting the material to be transplanted, implanted or transfused to treatment before and/or during and/or after transplant, implant or transfusion with one of the following components:
- a phosphatase having detoxifying activity for an endotoxin and/or for a derivative of endotoxin having endotoxic activity, said phosphatase preferably being alkaline phosphatase, more preferably human (alkaline) phosphatase and preferably being recombinant phosphatase;
- a derivative of phosphatase having phosphatase activity according to any of claims 1-14;
- a vehicle capable of delivering and/or inducing synthesis of said phosphatase and/or said derivative,
as such or as active component of a composition.

20. A pharmaceutical composition comprising at least one of the following components:

- an alkaline phosphatase having phosphatase activity in or on bone in vivo, more preferably human (alkaline) phosphatase and preferably being recombinant phosphatase;
- a derivative of an alkaline phosphatase having phosphatase activity in vivo according to claim 3, optionally in combination with any of claims 4-14;
- a vehicle capable of delivering and/or inducing synthesis of said phosphatase and/or said derivative,

as active component in vivo and further comprising a pharmaceutically acceptable carrier.

21. A pharmaceutical composition according to claim 20, wherein the active component is embedded in the lipid bilayer of a liposome, preferably in combination with negatively charged membrane constituents.

22. Use of at least one of the following components:
- an alkaline phosphatase having phosphatase activity in or on the surface of bone in vivo, more preferably human (alkaline) phosphatase and preferably being recombinant phosphatase;
- a derivative of alkaline phosphatase having phosphatase activity in vivo according to claim 3, optionally in combination with any of claims 4-14;
- a vehicle capable of delivering and/or inducing synthesis of said phosphatase and/or said derivative,

as active component in vivo for preparation of a pharmaceutical composition for prohylaxis or therapy of pathology requiring increased bone formation such as stimulating mending of broken bone and prophylaxis or therapy of osteoporosis.

23. A pharmaceutical composition comprising at least one of the following components:
- a substance for stimulating phosphatase activity of an endogenous phosphatase or a derivative thereof, said phosphatase or derivative having detoxifying activity for an endotoxin and/or for a derivative of endotoxin having endotoxic activity and/or said phosphatase or derivative having phosphatase activity in vivo in or on bone, said substance preferably being a substance for stimulating alkaline phosphatase activity, more preferably human (alkaline) phosphatase and preferably being recombinant phosphatase;

- a vehicle capable of delivering and/or inducing synthesis of said substance for stimulating phosphatase activity, more preferably human (alkaline) phosphatase and preferably being recombinant phosphatase,
as active component and further comprising a pharmaceutically acceptable carrier.

24. A pharmaceutical composition according to claim 23, wherein the substance for stimulating phosphatase activity is selected from one or more of the following:
an endotoxin, a substance having endotoxic activity, granulocyte colony stimulating factor (G-CSF), retinoic acid, a glucocorticoid.

25. A pharmaceutical composition according to claim 23 or 24 further comprising at least one of the following components:
- a phosphatase having detoxifying activity for an endotoxin and/or a derivative of endotoxin having endotoxic activity, said phosphatase preferably being alkaline phosphatase, more preferably human (alkaline) phosphatase and preferably being recombinant phosphatase;
- a phosphatase having phosphatase activity <u>in vivo</u> in or on bone, said phosphatase preferably being alkaline phosphatase, more preferably human (alkaline) phosphatase and preferably being recombinant phosphatase;
- a derivative of a phosphatase having phosphatase activity according to any of claims 1-14,
- a vehicle capable of delivering and/or inducing synthesis of said phosphatase and/or said derivative, as further active component.

26. Use of at least one of the following components:
- a substance for stimulating phosphatase activity of a phosphatase and/or of a derivative thereof, said phosphatase and/or said derivative having detoxifying activity for an endotoxin and/or for a derivative of endotoxin having endotoxic activity, said substance preferably being a substance for stimulating alkaline phosphatase activity, more preferably human (alkaline) phosphatase and preferably being recombinant phosphatase;
- a vehicle capable of delivering and/or inducing synthesis of said substance for stimulating phosphatase activity
as active component for preparation of a pharmaceutical composition for prophylaxis or therapy of pathology mediated by an endotoxin and/or by a derivative of endotoxin having endotoxic activity.

27. Use of at least one of the following components:
- a substance for stimulating phosphatase activity of an alkaline phosphatase, more preferably human (alkaline) phosphatase and preferably being recombinant phosphatase, and/or of a derivative thereof in vivo in or on bone;
- a vehicle capable of delivering and/or inducing synthesis of said substance for stimulating alkaline phosphatase activity in vivo
as active component for preparation of a pharmaceutical composition for prophylaxis or therapy of pathology requiring increased bone formation such as stimulating mending of broken bones and prophylaxis or therapy of osteoporosis.

28. Use according to claim 26 or 27, wherein the substance for stimulating phosphatase activity is selected from one or more of the following:
an endotoxin, a substance having endotoxic activity, granulocyte colony stimulating factor (G-CSF), retinoic acid, a glucocorticoid.

29. A method for preventing occurrence of a pathology mediated by an endotoxin and/or by a derivative of an endotoxin having endotoxic activity, said method comprising increasing endogenous alkaline phosphatase activity in a subject by administering at least one of the following components:
- a substance for stimulating phosphatase activity of an endogenous phosphatase and/or a derivative thereof;
- a vehicle capable of delivering and/or inducing synthesis of said substance for stimulating phosphatase activity
as such or as active component of a composition to the subject.

30. A method for preventing occurrence of a pathology mediated by an endotoxin and/or by a derivative of an endotoxin having endotoxic activity following transplant or transfusion, said method comprising subjecting the material to be transplanted or transfused to treatment activating the endogenous production of alkaline phosphatase of said material by administering at least one of the following components:
- a substance for stimulating phosphatase activity of an endogenous phosphatase and/or a derivative thereof, said phosphatase and/or derivative having detoxifying activity for an endotoxin and/or for a derivative of

endotoxin having endotoxic activity, said substance preferably being a substance for stimulating alkaline phosphatase activity;
- a vehicle capable of delivering and/or inducing synthesis of said substance for stimulating endogenous phosphatase activity
as such or as active component of a composition to the subject.

31. A method for activating production of alkaline phosphatase, preferably endogenous alkaline phosphatase in the body, the tissue or the body fluid of an animal or human comprising application of at least one of the following components:
- a substance for stimulating phosphatase activity in particular of an endogenous phosphatase and/or a derivative thereof, said phosphatase and/or derivative having detoxifying activity for an endotoxin and/or for a derivative of an endotoxin having endotoxic activity, said substance preferably being a substance for stimulating alkaline phosphatase activity;
- a vehicle capable of delivering and/or inducing synthesis of said substance for stimulating phosphatase activity
as such or as active component of a composition.

32. A method for prophylaxis or therapy of pathology requiring increased bone formation such as stimulating mending of broken bone and prophylaxis or therapy of osteoporosis, said method comprising administering at least one of the following components:
- an alkaline phosphatase having phosphatase activity in or on bone in vivo, more preferably human (alkaline) phosphatase and preferably being recombinant phosphatase;
- a derivative of an alkaline phosphatase having phosphatase activity in vivo according to claim 3 optionally in combination with any of claims 4-14;
- a vehicle capable of delivering and/or inducing synthesis of said alkaline phosphatase or derivative thereof;
- a substance for stimulating phosphatase activity of an alkaline phosphatase, more preferably human (alkaline) phosphatase and preferably being recombinant phosphatase and/or a derivative thereof in vivo in or on bone;
- a vehicle capable of delivering and/or inducing synthesis of said substance for stimulating endogenous phosphatase activity
as such or as active component of a composition to the subject.

33. A method according to any of claims 28-32, wherein the substance for stimulating phosphatase activity, said substance preferably being a substance for stimulating alkaline phosphatase activity is selected from one or more of the following:
an endotoxin, a substance having endotoxic activity, granulocyte colony stimulating factor (G-CSF), retinoic acid, a glucocorticoid.

34. A method of treatment of pathology associated with rapid bone formation such as osteosarcoma, said method comprising decreasing or inhibiting alkaline phosphatase activity, preferably in a target specific manner i.e. at the location where said pathology occurs.

35. A pharmaceutical composition comprising at least one substance capable of decreasing or inhibiting phosphatase activity and/or the concentration of phosphatase, in particular alkaline phosphatase (activity), said substance preferably being targeted to act at a location where undesired bone formation is to be prevented.

fig-1a

fig-1b

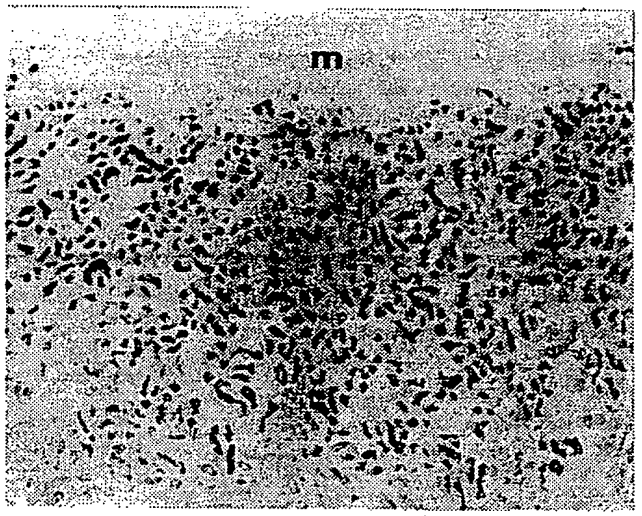


fig-1c

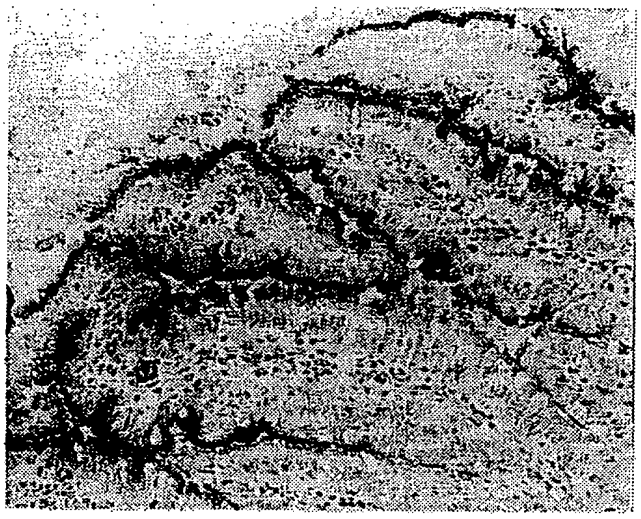


fig-1d

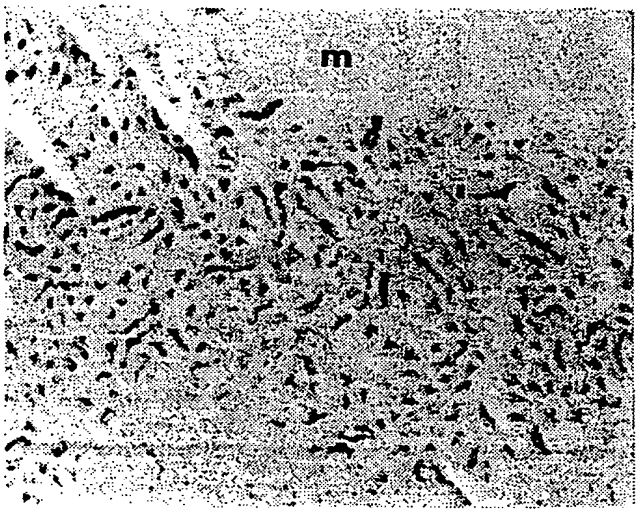



fig-1e

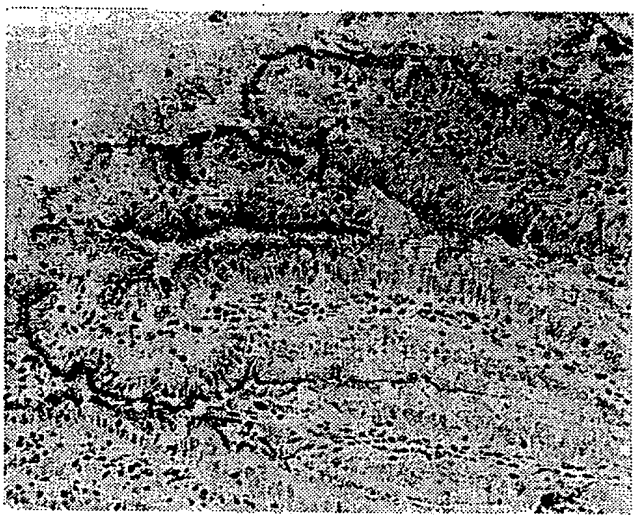


Fig - 1f

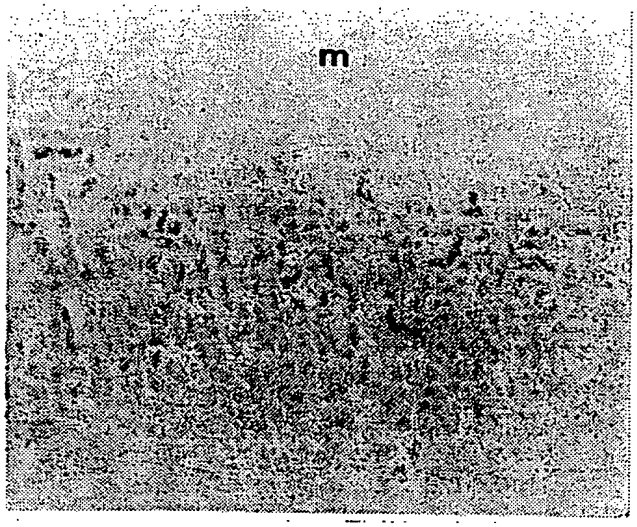



Fig - 4

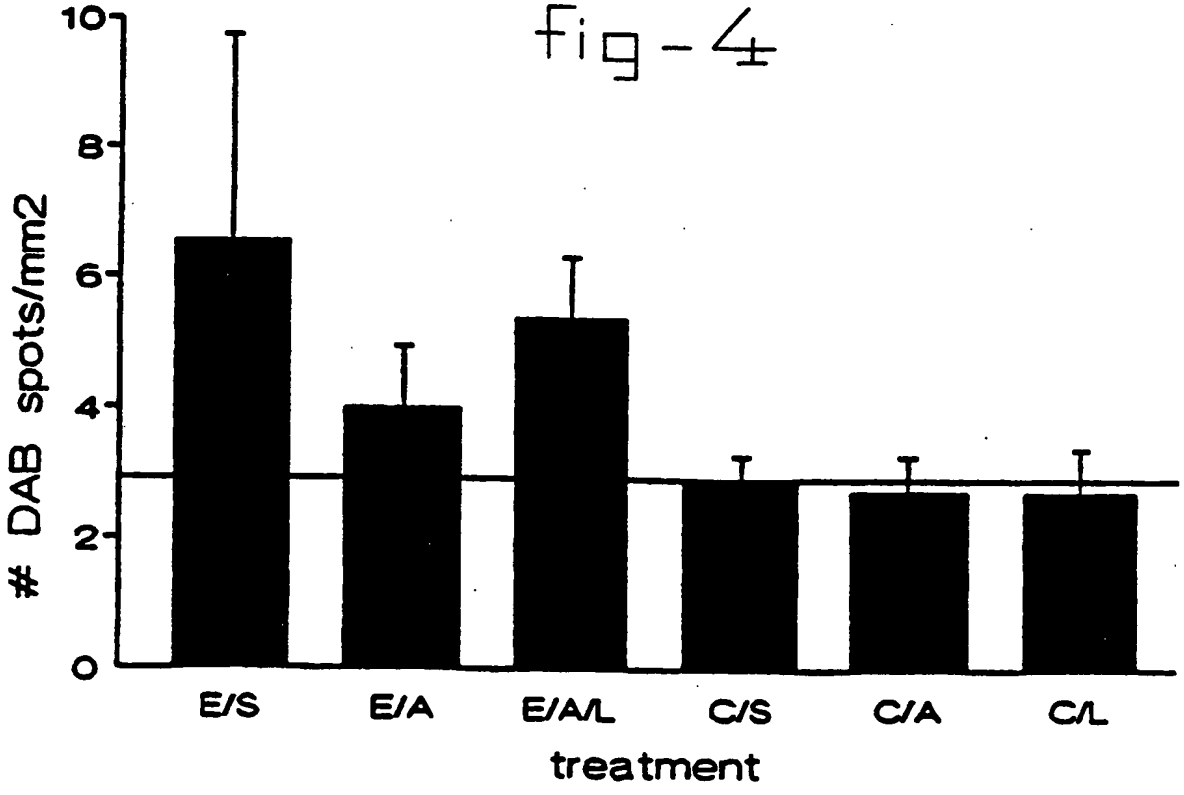


Fig - 2

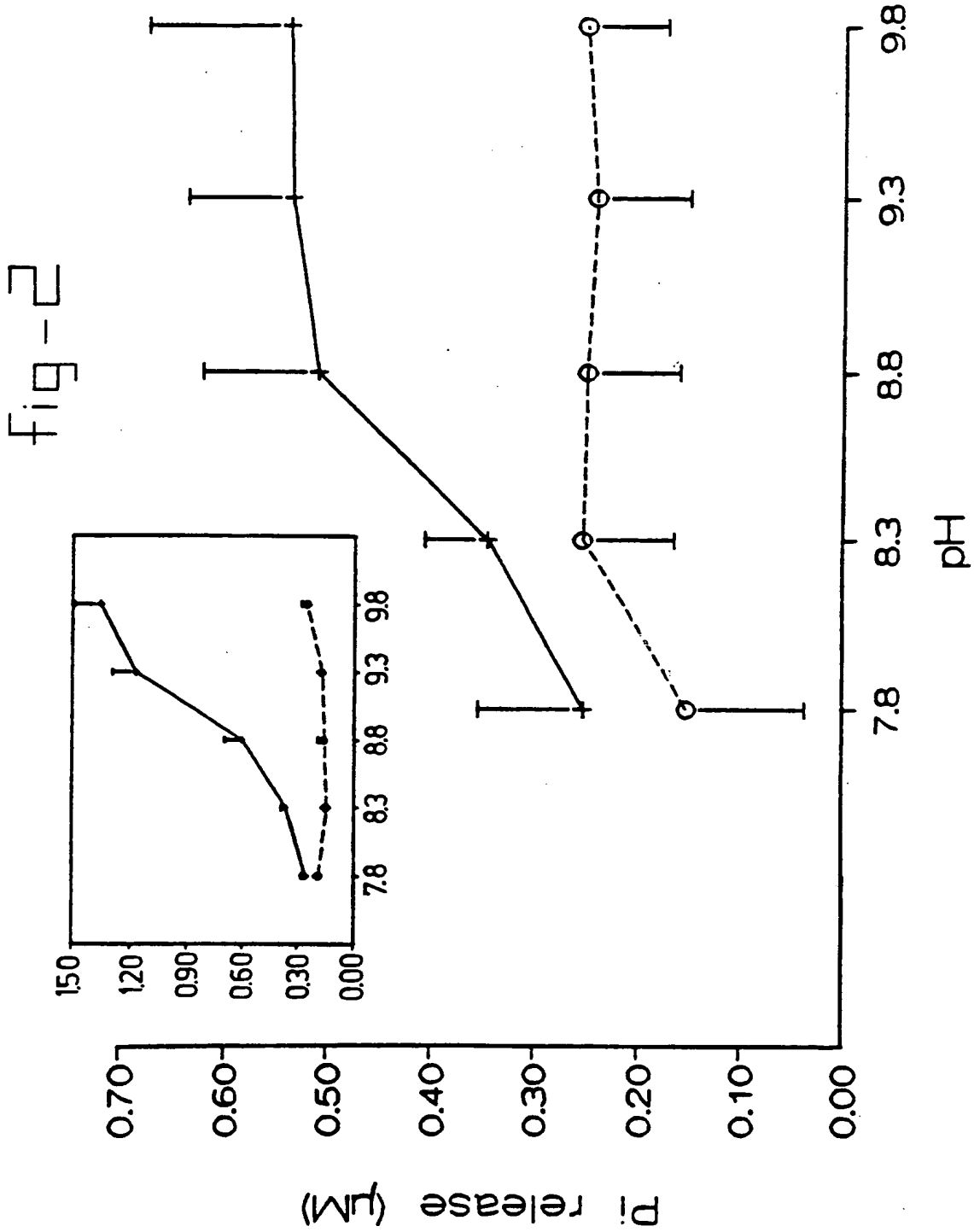


Fig - 3a

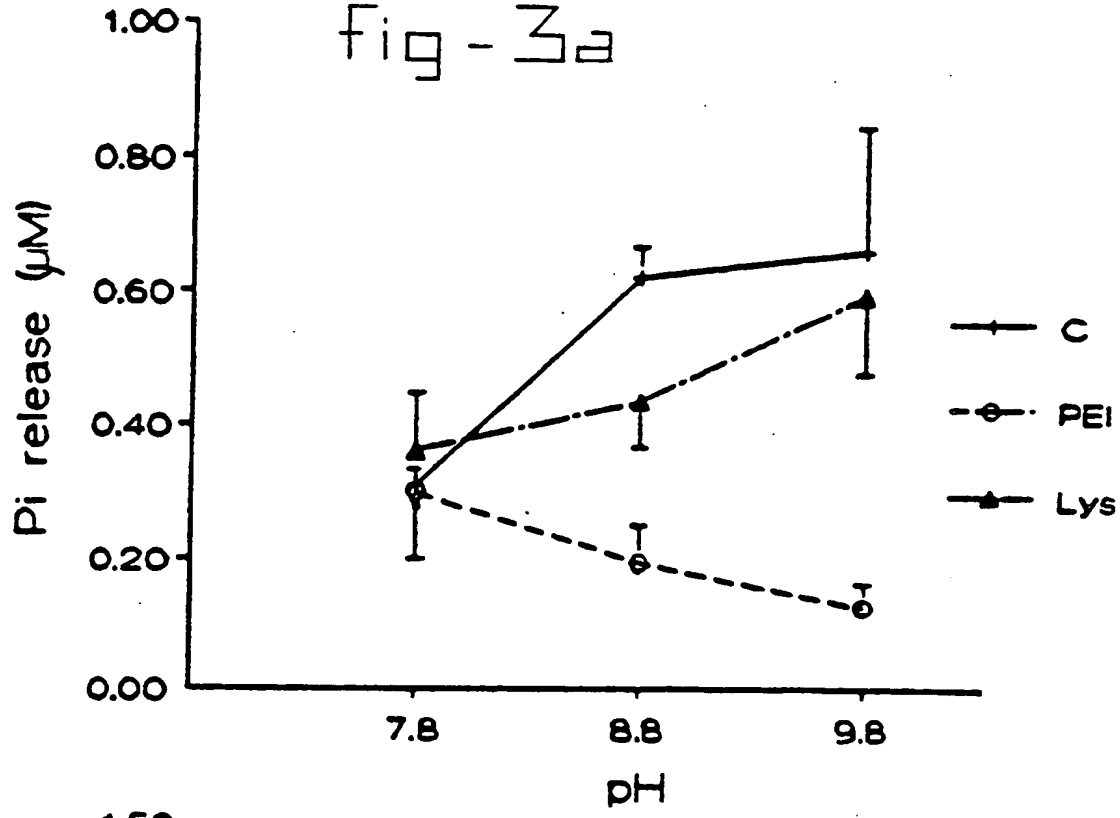



Fig - 3b

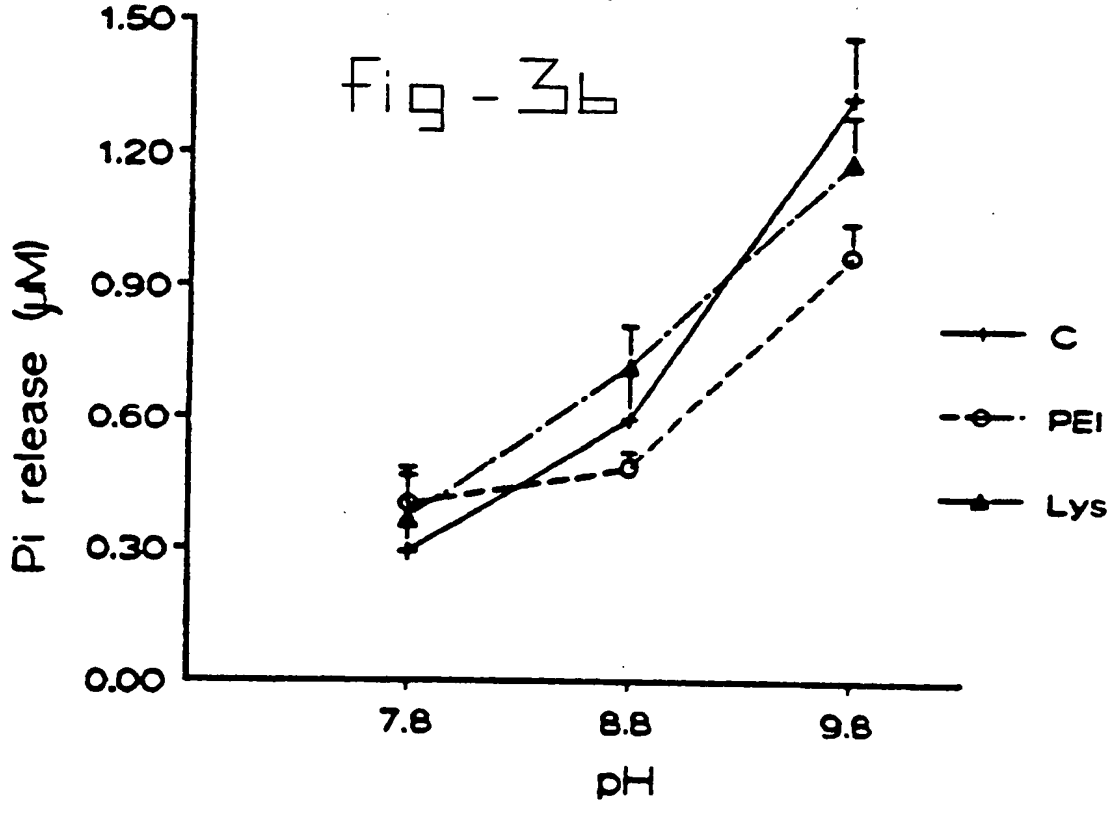


Fig-5

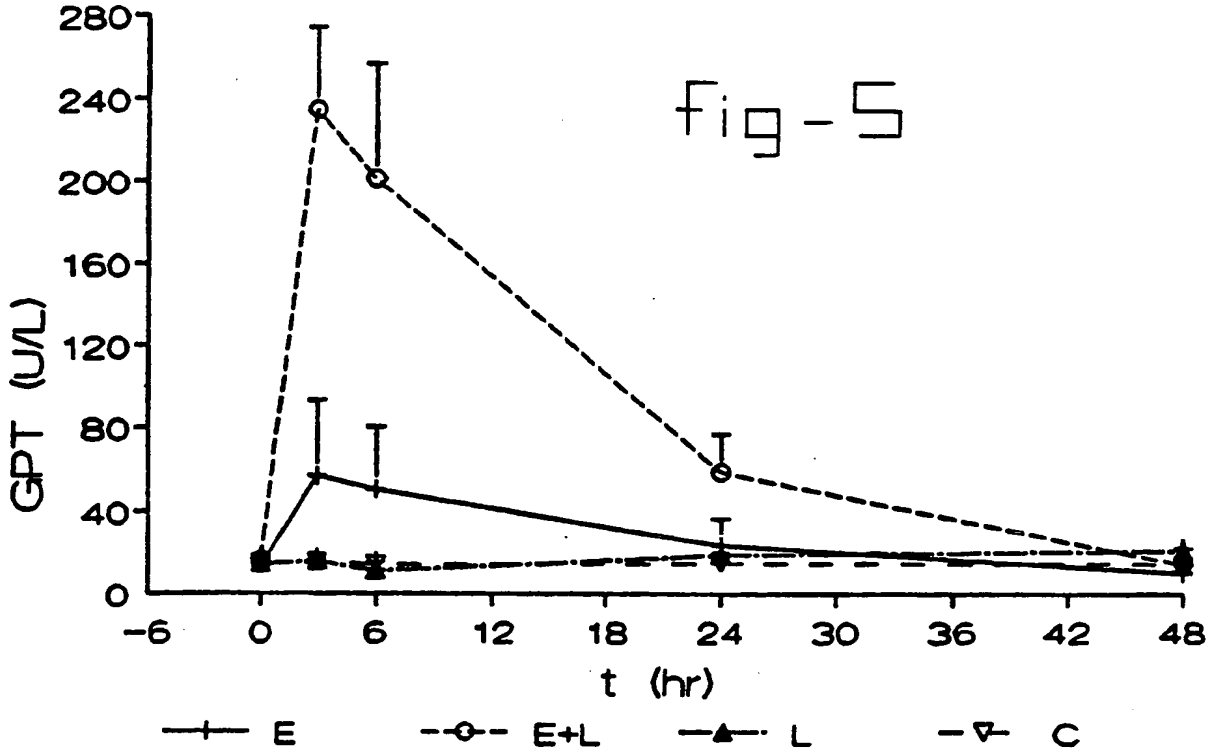
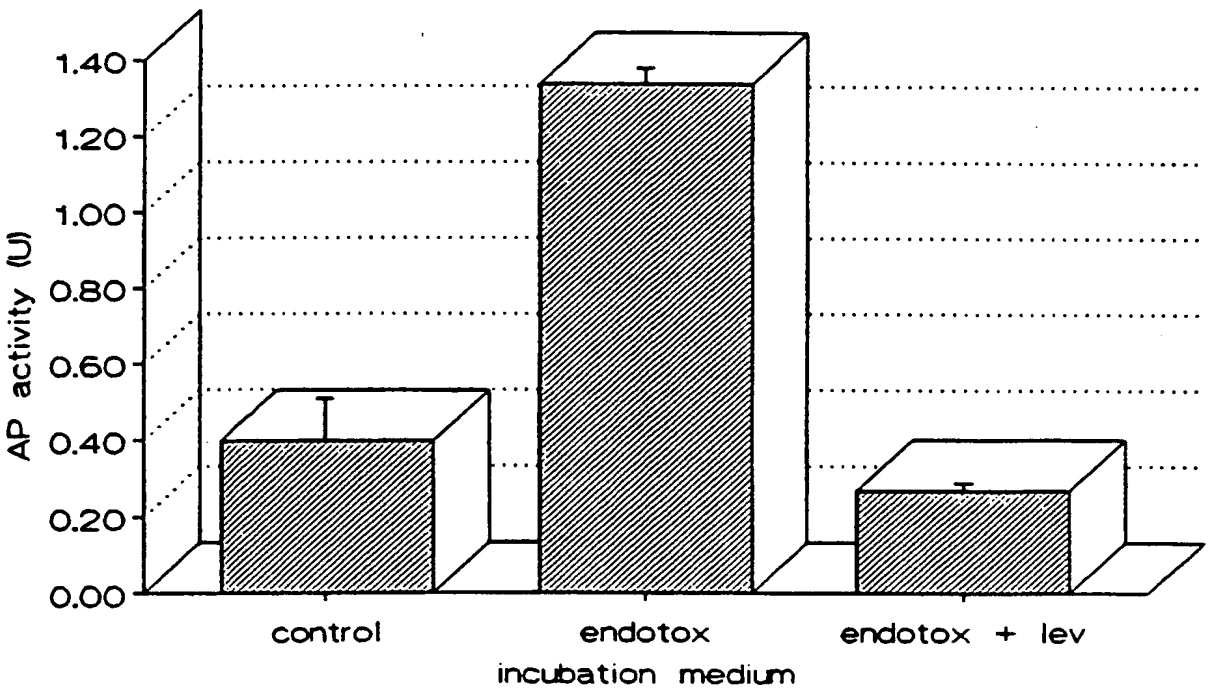



Fig-6




**SUBSTITUTE SHEET**

# INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 93/00171

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/16 A61K38/46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO,A,93 00935 (BRITISH TECHNOLOGY GROUP LTD.) 21 January 1993<br><br>see page 1, line 1 - page 8, line 13; claims 1-27<br>--- | 2,3,5,6, 8-10,15, 22,27,32 |
| X | US,A,4 409 332 (JEFFERIES ET AL.) 11 October 1983<br><br>the whole document<br>---<br>-/-- | 2-10,15, 17-20, 22,23, 25,27,32 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 14 April 1994 | 20. 05. 94 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+ 31-70) 340-3016 | Hornig, H |

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | US,A,4 394 370 (JEFFERIES) 19 July 1983<br>see column 2, line 20 - line 24<br>see column 5, line 17 - line 25; claims 1-12 | 2-8,10, 15, 17-20, 22,23, 25,27,32 |
| X | EP,A,0 441 252 (ABBOTT LABORATORIES) 14 August 1991<br>see page 5, line 8 - line 11; table 1 | 1-8,12 |
| X | EXPERIMENTAL HEMATOLOGY<br>vol. 20, no. 4 , May 1992 , SPRINGER VERLAG, NEW YORK, US;<br>pages 388 - 390<br>G. CHIKKAPPA 'Control of neutrophil alkaline phosphatase synthesis by cytokines in health and diseases'<br>cited in the application<br>the whole document | 15,23-25 |
| X | BLOOD<br>vol. 76, no. 12 , 15 December 1990 , SAUNDERS, NEW YORK, US;<br>pages 2565 - 2571<br>A. RAMBALDI ET AL. 'Expression of leukocyte alkaline ohosphatase gene in normal and leukemic cells: Regulation of the transcript by granulocyte colony-stimulating factor'<br>cited in the application<br>the whole document | 15,23-25 |
| X | BLOOD<br>vol. 70, no. 2 , August 1987 , SAUNDERS, NEW YORK, US;<br>pages 404 - 411<br>A. YUO ET AL. 'Recombinant human granulocyte colony-stimulating factor repairs the abnormalities of neutrophils in patients with myelodysplstic syndromes and chronic myelogenous leukemia'<br>cited in the application<br>the whole document | 15,23-25 |
| A | WO,A,92 15316 (RIJKSUNIVERSITEIT TE GRONINGEN) 17 September 1992<br>cited in the application<br>see page 2, line 24 - page 10, line 18 | 1-14 |

2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL93/00171

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claims 22,27,28(as far as they concern in vivo treatment of human or animals) and claims 18,19,29,31-34 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/NL 93/00171

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| WO-A-9300935 | 21-01-93 | NONE | | |
| US-A-4409332 | 11-10-83 | CA-A- | 1186625 | 07-05-85 |
| US-A-4394370 | 19-07-83 | US-A- | 4472840 | 25-09-84 |
| EP-A-0441252 | 14-08-91 | AU-A- | 7083791 | 08-08-91 |
| | | CA-A- | 2035826 | 08-08-91 |
| | | JP-A- | 4254315 | 09-09-92 |
| | | JP-A- | 4349881 | 04-12-92 |
| WO-A-9215316 | 17-09-92 | NL-A- | 9100434 | 01-10-92 |
| | | AU-A- | 1566392 | 06-10-92 |
| | | EP-A- | 0575432 | 29-12-93 |